

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/92293 A2

- (51) International Patent Classification⁷: **C07K 1/00**
- (21) International Application Number: PCT/NL01/00422
- (22) International Filing Date: 31 May 2001 (31.05.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
09/583,426 31 May 2000 (31.05.2000) US
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: NANO-CRYSTALLOGENESIS, METHODS FOR MAKING CRYSTALS, COMPOSITIONS COMPRISING THEM AND USES THEREOF

(57) Abstract: The invention provides methods and means for identifying crystallisation conditions for biomolecules such as proteinaceous substances and nucleic acids. The invention further provides methods for crystallising such molecules under optimal conditions, as well as compositions comprising crystals produced by these methods with improved properties for pharmaceutical use, for structure identification and for further methods for producing crystals.



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Title: Nano-crystallogenesis, methods for making crystals, compositions comprising them and uses thereof.

The present invention relates to methods for identifying crystallization conditions for biomolecules using e.g. a method and an apparatus for screening the phase behaviour in liquid gel or solid phase of such biomolecules, such as proteins, peptides, macromolecules or complexes thereof. The present
5 invention also allows the screening of crystallization conditions that cannot be tested in conventional ways, i.e. in larger volumes and also allows crystals of a higher quality to be grown. The present invention furthermore relates to the parallel detection of phase transitions, more specific crystallization, of proteins and other bio-macromolecules or biomolecular complexes. The present
10 invention also provides methods for producing crystals of biomolecules on any desired scale using the conditions identified according to the invention. Further uses of the crystals and compositions comprising such crystals are also provided. Crystals of biomolecules according to the invention can also be used to collect information on the structure of such biomolecules. As the method
15 reduces gravity-induced convection during crystal growth, the crystalline order of crystals produced with the method will approach that of high quality crystals grown in space, i.e. in microgravity conditions.

Collecting information related to the structure of proteins and other biomolecules is becoming an important field of technology. With information
20 on the three-dimensional structure of biomolecules, the knowledge on the fundamental processes of life can be further understood and working mechanisms of essentially biological processes can be elucidated or better understood. This knowledge may allow for the more efficient synthesis of bio-active compounds and the optimization of the development of pharmaceuticals.
25 Other uses of such knowledge can be found in the development of alternatives for growth hormones and for enzymes, for instance for use in cleaning compositions.

Bulk states of matter (phases) can be gaseous, liquid and solid. It is the bulk state of matter that determines macroscopic physical characteristics of a biomolecule. Within the solid state, different solid phases can be distinguished. For instance a solid can be amorphous or have various crystalline states such
5 as is known for carbon, which can be in the form of graphite, diamond or buckminsterfullerene.

Crystallization is in general considered as the separation or precipitation out of a liquid environment. The basic approach to crystallization is usually fairly simple. The biomolecule to be crystallized is dissolved or
10 suspended and subsequently subjected to conditions that affect the solubility of the biomolecule in solution. This can be achieved by removal of the solvent or by the addition of other compounds that reduce the solubility, optionally in combination with variation of other factors such temperature, pressure or gravitational forces. When the conditions are right the crystals will separate.
15 For biomolecules that have a complex chemical composition, the separation out of solution is in general applicable. However, the relations between the conditions and the crystallization are generally not well understood. The optimization of the crystallization conditions is therefore mainly based on trial and error.

20 Several different types of techniques can be distinguished in protein crystallogenesis (i.e. the growing of crystals). Most prominent are the hanging drop and the sitting drop method, batch crystallization, micro-dialysis and free interface diffusion (see for an overview of techniques Ducruix, A. & Giegé, R. (1992) 'Methods of crystallization' in 'Crystallization of nucleic acids and
25 proteins', Oxford University Press, ISBN 0-19-963246-4, pp. 73-98). In these methods a supersaturated protein solution is created by diffusion of components such as precipitants and solvents to or from said solution. A major disadvantage of these methods is that they require relatively large amounts of material (at least 10 microgram is required per test of one particular
30 condition). Furthermore, only the temperature can usually be changed during

an experiment. Other vital parameters, like the rate of evaporation, or the final concentration of the protein, are much more difficult to manipulate during the experiment, if at all.

The crystallogenesis of biomolecules is generally affected by parameters
5 such as concentration, pH of the solution, buffer strength and type of buffer, ionic strength and ionic species, type of precipitant, surfactants, additives and complexants such as cofactors and inhibitors, etc. Next to the chemical composition of the mother liquor, the temperature is a vital parameter. The total volume in which crystallization is induced or allowed to take place, is
10 generally not assumed to be a relevant parameter determining whether or not crystallization happens. However, the present invention establishes that in very small volumes (100 nL and below) better crystals can be grown. Also it is established that in such small volumes more highly supersaturated solutions can be tested for the growth of crystals. Such highly supersaturated solutions
15 would immediately result in rapid (non-crystalline) precipitation in larger volumes (1 mL and above), rendering these conditions in larger volumes useless for growing high quality crystals.

For proteins, the optimal temperature for crystal growth is usually between -15 and 40 °C. Many proteins do not crystallize at all, unless within
20 10 °C of their optimum temperature. The quality of protein crystals is affected by gravity induced convection resulting from concentration gradients that are generated during crystallization, so in many cases, better crystals can be grown in space under microgravity conditions. Crystals of some proteins can appear within a day, but for other proteins crystallogenesis can take months.
25 Also the set-up of the crystallization experiment can be important for the emergence or quality of crystals. Vapor diffusion techniques are very popular, but also batch crystallization, free interface diffusion and micro-dialysis, each of these techniques either in solution or in a gel, have been used successfully. The state of the art is summarized in A. McPherson (1998) 'Crystallization of
30 Biological Macromolecules', Cold Spring Harbor Laboratory Press, ISBN 0-

87969-527-7 and in T. Bergfors (1999) 'Protein Crystallization Techniques, Strategies and Tips', IUL Biotechnology Series, ISBN 0-9636817-5-3.

Due to the large number of parameters that affect crystallogenesis, determining the optimal combination of parameters results in an
5 overwhelming amount of possible combinations. Linear optimization (testing one condition after the other) based on trial and error is hence a long and cumbersome method, if at all feasible. Moreover, in many cases biomolecules are not readily available in quantities that allow for crystallization, let alone for testing numerous conditions for crystallization.

10 It is therefore an object of the present invention to provide methods in which testing of many combinations is possible and can be done efficiently and rapidly and preferably in parallel, i.e. testing various combinations at the same time.

Determining the conditions under which crystallogenesis of
15 biomolecules is effectively performed suffers further from another drawback, namely the earlier mentioned limited availability of the biomolecules. More often than not biomolecules are available only in minute quantities (microgram or even nanogram quantities), generally obtained after long and tedious isolation and purification processes or after complex and laborious
20 syntheses.

The problem of the limited availability of biomolecules is at present solved by devising ways and means of obtaining larger amounts of the biomolecule of interest, but this will work only for a limited number of biomolecules.

25 Accordingly, there is a need for a method that allows for the rapid screening of possible conditions that are suitable for the crystallogenesis of biomolecules and which method can be used with small (sub-microgram) amounts of biomolecules.

The present provides in one aspect a method wherein the multi-
30 dimensional phase behaviour in general and more in particular

crystallogenesis conditions can be rapidly screened in parallel, thereby using only small amounts of biomolecules.

The present invention thereby provides a method for identifying crystallization conditions for at least one species of biomolecule, comprising the provision of a set of compositions of a volume of 1-100 nanoliter, each said composition comprising at least one said species of biomolecule, and inducing or allowing each said composition to adopt at least a first condition possibly influencing said crystallization and detecting any crystallization in at least one composition. According to the invention, preferably a plurality of compositions is provided, each differing in at least one component that influences the crystallization behaviour of the biomolecule to be crystallized. Biomolecules according to the invention are nucleic acids, DNA's, RNA's, PNA's, polypeptides, peptides, glycoproteins and other proteinaceous substances, lipoproteins protein-nucleic acid complexes, carbohydrates, biomimetics, etc. Although the art teaches that large quantities of material are required for successful crystallization we have shown that sub-microgram quantities can be used. Because of the small volumes that can be used in the methods according to the invention, availability of biomolecules is less of a problem and rapid testing of numerous conditions and easy adjustment of relevant conditions is easily obtained.

The difference from one composition to the next may be a difference in physical, chemical or both conditions. The conditions influencing crystallization of biomolecules in general are well known and apply also on the sub-micro level. Further explanation is thus hardly necessary for the person skilled in the art. However hereunder we provide a list of chemicals that – when present in the mother liquor – possibly influence crystallization of biomolecules. The list is of course not exhaustive. The chemical composition of the mother liquor is essential for proper crystallization. A very important determinant is the pH and the buffering component of the solvent: common buffering components that have been used include N-[2-acetamido]-2-

aminoethanesulphonic acid (pH range 6.1 to 7.5), N-[2-acetamido]-
2iminodiacetic acid (pH range 6.0 to 7.2), 3-[(1,1-dimethyl-2-
hydroxyethyl)amino]-2-hydroxypropanesulphonic acid (pH range 8.3 to 9.7),
N,N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid (pH range 6.4 to 7.8),
5 N,N-bis[2-hydroxyethyl]glycine (pH range 7.6 to 9.0), bis[2-
hydroxyethyl]iminotris[hydroxymethyl]-methane (pH range 5.8 to 7.2), 1,3-
bis[tris(hydroxymethyl)methylamino]propane (pH range 6.3 to 9.5), 4-
[cycloacylamino]-1-butanephosphonic acid (pH range 10.0 to 11.4), 3-
cyclohexylamino]-1-propanesulphonic acid (pH range 9.7 to 11.1), 3-
10 [cyclohexylamino]-2hydroxy-1-propanesulphonic acid (pH range 8.6 to 10.0),
citric acid (pH range 2.2 to 6.5), N,N-bis(2-hydroxyethyl)glycine (pH range 8.6
to 10.6), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (pH range
6.8 to 8.2), imidazole (pH range 6.2 to 7.8), 2-[N-morpholino]ethanesulphonic
acid (pH range 5.5 to 6.7), 3-N-[morpholino]-2-hydroxypropanesulphonic acid
15 (pH range 6.2 to 7.6), piperazine-N,N-bis[2-ethanesulphonic acid] (pH range
6.1 to 7.5), sodium acetate (pH range 3.6 to 5.6), sodium cacodylate (pH range
5.0 to 7.4), sodium citrate (pH range 5.6 to 7.5),
tris(hydroxymethyl)aminoethane (pH range 7.0 to 9.0), triethanolamine (pH
range 7.3 to 8.3), N-tris[hydroxymethyl]methylglycine (pH range 7.4 to 8.8),
20 but also many other organic and inorganic buffering components haven been
used. Counter ions to these buffers include sodium, lithium, potassium,
chloride, sulphate, phosphate, ammonia, and many others. Salts that are
commonly included in crystallization experiments are: the acetate, citrate,
formate, chloride, and tartrate salts of sodium, potassium, lithium,
25 ammonium, magnesium, calcium or zinc, in concentrations from 0.01 to
saturation (up to 8 M), but other salts have also been used. Precipitants that
are commonly included in crystallization experiments are: polyethylene glycol
in molecular weights of 200, 400, 1000, 1500, 3350, 4000, 6000, 8000, 10000,
20000, in concentrations between 2% (w/w) to 40% (w/w), diethyloxide (2% to
30 40% w/w), iso-propanol (2% to 40% w/w), ethylene glycol (2% to 40% w/w), 1,6

hexanediol (0.2 to 4 M), (+/-)-2-methyl-2,4pentanediol (2% to 50% w/w), ammonium sulphate (0.2 to 4 M), sodium chloride (0.2 to 5 M), but many others have also been used. Next to these components, cofactors and other additives can be included. Commonly used are salts of barium, cadmium, copper, cobalt, magnesium, manganese or zinc, polyamines like spermine, or spermidine, chelators like EDTA, nucleotides like guanine or adenosine, diphosphate or triphosphate. There are at least 70 of such additives that are routinely being used. The concentration of the additives is usually between 0.1 mM and 50 mM. Many proteins do not crystallize at all, unless all the parameters are right to within 10%.

An easy way of providing a set of compositions for determining crystallization conditions for biomolecules is a method wherein said set of compositions is provided on a solid support medium comprising of a multitude of separate test positions, each comprising one composition. These separate test positions may be, but are not restricted to separate cells, containers, wells, or other depressions in the solid support. Thereby an array-like test format is provided allowing for easy automation of dispensing compositions and/or changing conditions per composition and of detection of phase change behaviour (typically crystallization of the biomolecule). In any format the crystallization may be best achieved by a further change to the conditions of the original starting compositions. Such further changes in conditions can also be applied in the present invention and very conveniently so in the array format. Further changes may also be used to fine-tune the crystallization. It is often desired to be able to influence the speed and duration of crystallization processes, as well as the size and size distribution and homogeneity and/or purity of resulting crystals. In order to do so the invention is particularly suitable. A fast formation of nuclei can be designed, followed by slow crystallization on those nuclei, if desired. Conditions on how to influence speed and growth and how to end crystallization can all be determined. After that a production scale of crystals, for instance for pharmaceutical use (in

pharmaceutical preparations) can be based on the conditions identified according to the invention.

Thus the invention also provides a method for producing a composition comprising crystals of a biomolecule of a narrow size distribution, comprising
5 selecting crystallization conditions for said biomolecule by a method according to the invention and scaling up said conditions to a desired total volume, where crystals obtained by the method can be used as nucleation seeds. The composition that is the result of such a method will be more homogeneous with respect to the size and composition of the crystals then available until now.

10 Thus the invention also encompasses these compositions. Such compositions can be used for pharmaceutical purposes or as seed crystals in further crystallization processes. In such an embodiment the invention provides a method for producing crystals of a biomolecule, comprising providing an over-saturated composition of said biomolecule and providing said over-saturated
15 composition with a composition of seed crystals according to the invention. A preferred use of the crystals produced according to the invention is in the determination of structures of biomolecules. Thus the invention further, provides a method for determining the structure of a biomolecule comprising subjecting a composition or a crystal from said composition according to the
20 invention to electron diffraction and/or X-ray diffraction and determining its structure.

The invention further provides as a specific embodiment a method for screening the phase behaviour of at least one species of biomolecule comprising the steps of:

25 providing a solid support medium comprising of a multitude of separate test positions,

combining the biomolecule in each separate cell with one or more other components preferably such that the total composition of an individual test position differs in at least one aspect from the composition of at least one
30 other test position, where the mixing of components and biomolecules is either

simultaneously or in sequence, in any order;

optionally modifying the conditions that lead to changes in phase behaviour in individual compositions;

detecting changes in phase behaviour of the compositions in the
5 separate test positions.

Additionally to speed up the identification of ideal conditions, the different cells can be compared (automatically) to each other.

Of course the change in phase behaviour of the composition is preferably mainly caused by a phase change of the biomolecule.

10 To enhance crystallization of the biomolecule an inert nucleus for crystallization may be provided. In one embodiment the invention thus provides a method wherein the surface of the support medium comprises at least one crystallization nucleus comprising an inert crystal, preferably those wherein the nucleation centers are in the form of small crystallites preferably
15 of salts or complexes of the biomolecule.

The crystallization initiation may further be influenced by a step in which nucleation is induced by addition of nucleation centers, by agitation, vibration, micro-wave treatment, (ultra)sonic treatments or a combination thereof.

20 Detecting changes of phase behaviour is generally within the skill of the art. Typical methods are those wherein changes in phase behaviour are detected by measuring, continuously or intermittently, optical and/or diffraction characteristics of the individual cells.

Dispensing methods suitable for the present invention are piezo-electric
25 dispensing techniques, bubble jet dispensing techniques, electrospray dispensing techniques, micro- and nano- dispensing techniques, flood filling or combinations thereof.

The cells on the solid support can be further equipped with means for controlling the atmosphere in or directly above the cells. To this end the
30 support medium is for instance fitted with sealing devices that seal off the

entire support or seal off individual cells or groups of cells. To this end balls, plates, caps, films, oils etc. can be provided. The art teaches that when the sealant is a liquid that is immiscible with the composition of the tests, said compositions can actually be dispensed through the sealant in order to
5 completely prevent or slow down evaporation (Patent GB2249492, Douglas Instruments). The present invention provides that piezo-electric dispensing techniques, bubble jet dispensing techniques, electrospray dispensing techniques, micro- and nano- dispensing techniques are particularly suitable for dispensing said compositions through the sealant in order to completely
10 prevent or slow down evaporation.

Accordingly, the present invention further provides for a method for screening the phase behaviour of at least one biomolecule comprising the steps of:

providing a solid support medium comprising of a multitude of
15 separate test positions;

providing two or more of the separate test positions with a solution of the biomolecule and other components such that the total composition at an individual test position differs in at least one aspect from the composition of at least one other test position and the total composition at
20 the test position has a volume of at most 100 nanoliter;

optionally modifying the conditions that lead to changes in phase behaviour in individual compositions;

detecting changes in phase behaviour of the compositions in the separate test positions.

25 The major advantages of the method according to the invention are that automated set-up of the experiments is generally quicker for small volumes, the automated detection of crystals in an array of conditions is quicker as more samples can be tested simultaneously, less material is required, thereby reducing wastage, more tests can be performed given the amount of material
30 available, and the chance that the conditions under which crystallization is

achieved are identified significantly increases. A further advantage is that these small volume tests often provide single nucleation events, resulting in virtually all of the protein in the test ending up in one single crystal. Finally, in these very small volumes, gravity-induced convection is minimized, leading to crystals with fewer growth defects. In a preferred embodiment of the invention, such high-quality crystals are used as seeds in scale-up experiments to produce larger high quality crystals suitable for structure determination. This eliminates the necessity of growing crystals under microgravity, which is a very costly procedure.

In a preferred embodiment, the volume of the total composition in the test position is less than 50 nanoliter, 1 nanoliter, 100 picoliter, or even less than 10 picoliter.

In a preferred embodiment of the invention the screening of the phase behaviour is carried out on a solid support medium comprising a multitude of cells (wells, containers of other depressions), each cell having a volume of less than 100 nanoliter. In preferred embodiments of the invention, the cells have volumes that are less than 50 nanoliter, 1 nanoliter, 100 picoliter, or even less than 10 picoliter. Preferably the cells of the solid support are in the form of an array.

In one aspect of the invention, there is provided a solid support medium. This is devised by methods such as molding of a silicon rubber, shaping a photoresist resin by lithographic techniques prior or after layering on a suitable support, molding various plastics, etching metals or glass or by any other means known in the art for providing a support with pits, wells or depression structures of the desired size, corresponding to the desired volume of the cells. Preferably, the solid support medium is coated, preferably with a hydrophilic or hydrophobic coating.

The solid support contains cells, preferably in an array (such as is known for instance from microtiterplates), in an amount preferably exceeding 400 positions, preferable more than 1000, more preferable more than 2500

positions. As the volume of the cells is small, these amounts of cells are provided on a relative small surface area. In a preferred embodiment, the solid supports according to the invention are provided with more than 400 cells per square centimeter, preferably more than 1000, more preferably more than
5 2500 cells per square centimeter.

The cells on the solid support are preferably further equipped with means for controlling the atmosphere in or directly above the cells. To this end the support medium is for instance fitted with sealing devices that seal off the entire support or seal off individual cells or groups of cells. To this end balls,
10 plates, caps, films etc. can be provided. In another preferred embodiment, the sealant is a liquid that is immiscible with the composition of the tests, and preferably the compositions are dispensed through said sealant in order to completely prevent or slow down evaporation.

Biomolecule in the definition of the present invention pertains to all
15 matter of which the phase behaviour is to be determined. Within the scope of the present invention this means compounds such as biomolecules, proteins, peptides, nucleic acids, enzymes, hormones, biomimetics and mixtures and complexes thereof and chemically or otherwise modified forms thereof. The biomolecule may be provided in a pure form or not in a pure form, for instance
20 as a part of a mixture. It is preferable that the biomolecule is in substantially pure form.

The biomolecule of which the phase behaviour is to be determined is preferably provided on the solid support in the form of a crystalline or amorphous solid material or otherwise aggregated or solid state or in the form
25 of a solution, emulsion or suspension. The biomolecule is preferably coated or deposited directly, or through a spacer, on the optionally coated, surface of the cells.

The biomolecule is brought in the cells of the solid support by techniques that allow for the precise and controlled delivery of minute
30 amounts of matter. Suitable techniques are those that allow for the precise

and controlled delivery of amounts of liquids or solids in the range of microliters or - grams, preferably nanoliters or - grams, more preferable picoliters or - grams. When the biomolecule is provided in any liquid form, preferably dispensing techniques such as piezo-electric dispensing techniques, bubble-jet dispensing techniques, electrospray dispensing techniques, as well as other micro- and submicro-dispensing techniques can be used and preferably nano-dispensing techniques are used. By using these techniques for dispensing it is possible to provide each cell with a predetermined amount of biomolecule. Hence, it is likewise possible to provide to each cell a different amount of biomolecule.

Also none-directional dispensing of a fluid onto the substrate, for instance by flood-filling by submerging the substrate into a fluid, pouring the fluid over the substrate, or other flood-filling techniques can be employed advantageously.

When the biomolecule is provided by means of some carrier, for instance in the form of a solution or emulsion, it is a preferred option to remove at least partly solvents and/or other carrier fluids by allowing or causing a degree of evaporation, drying, draining, lyophilisation or other fluid removal technique from the cells.

The biomolecule is present in the cell in an amount of up to 100 nanogram, preferably up to 10 nanogram. In general the cells are filled up to the complete volume, however, it is likewise envisaged that only part of the cell is filled, as long as the total volume in the cell of the biomolecules and other added or removed components can be adequately established.

Prior to the addition of the biomolecule, after the addition of the biomolecule, or concomitantly therewith, the cells are provided with other components or combinations of other components. These components are selected from the group consisting of buffers, salts, surfactants, solvents, chaotropic agents, precipitants, cofactors, inhibitors as discussed above. The components are added preferably in increasing or decreasing amounts or

strengths. For example, a salt is added in an increasing concentration and its influence on the phase behaviour is determined or a pH is gradually increased over a number of cells, while the other parameters are kept constant or are also varied in a predetermined manner. Here an example is given of a comprehensive screening is given, where pH, type and concentration of precipitant are varied. For instance, an array of which the cells are filled with a biomolecule, contains combinations of:

- a main buffering component of a concentration between 1 mM and 1 M, with a pH varying over the array in steps of 0.5 pH units or smaller: formate (pH 3.0 to 4.0), citrate (pH 3.5 to 4.5), acetate (pH 4.0 to 5.5), MES (2-[N-morpholino]ethanesulphonic acid) (pH 5.0 to 7.0), HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) (pH 6.5 to 8.0), Tris (tris(hydroxymethyl)aminoethane) (pH 7.5 to 8.5), Bicine (N,N-bis[2-hydroxyethyl]glycine) (pH 8.0 to 9.5) and CAPS (3-cyclohexylamino]-1-propanesulphonic acid) (pH 9.0 to 10.5), but other buffers may also be used;
- a precipitant the final concentration of which is varied over the array in at least 6 steps between the lowest and highest concentration: ammonium sulphate (0.6 M to 1.6 M), sodium chloride (0.75 M to 2 M), lithium chloride (1.5 to 4 M), sodium potassium tartrate (0.24 M to 0.64 M), polyethylene glycol MW 400 (15% w/v to 40 % w/v), polyethylene glycol MW 2000 (7.5% w/v to 20% w/v), polyethylene glycol MW 4000 (7.5% w/v to 20% w/v), polyethylene glycol MW 8000 (7.5% w/v to 20% w/v), polyethylene MW 20,000 (4.5% w/v to 12 % w/v), Jeffamine M-600 (7.5% w/v to 20% w/v), ethylene glycol (15% v/v to 40% v/v), dioxane (7.5% v/v to 20% v/v), but other precipitants may also be used.

In a preferred embodiment, the method comprises an additional step wherein nucleation is induced by addition of nucleation centers, by agitation, vibration, micro-wave treatment, (ultra)sonic treatments or a combination thereof. Preferably nucleation centers are added in the form of small crystallites, preferably in the form of salts or complexes of the biomolecule. Other preferred nucleation centers are zeolites or insoluble minerals or oxides.

These nucleation centers are preferably added separately or in combination with the biomolecule or any other of the components or be suspended therein. The centers are preferably provided in the cell, for instance through adhesion or coating of the nucleation center thereupon.

5 The array of cells is optionally subjected to modified conditions such as an increasing or decreasing temperature, pressure, vapor pressure or gravitational field (centrifugal forces). It is preferred to provide for sufficient time to allow for crystallization to take place. Also the atmosphere is preferably controlled. Control of the atmosphere is preferably achieved by
10 providing an inert atmosphere above the cells, the vapor pressure of which is carefully controlled. The vapor pressure of the atmosphere will influence the concentration of the biomolecule in the cell and the phase behaviour thereof. In a preferred embodiment, the concentration of the biomolecule in the composition is modified by equilibrating the composition against a gas or a
15 fluid with a different vapor pressure. Other ways of controlling the evaporation of liquids from the cells or to seal cells from the outside atmosphere are appropriately shaping the wells into which the liquids are dispensed, with openings smaller than, or equal to, 1/6th of the total surface of the liquid that can be contained by the well if filled to the brim; dispensing the volumes
20 through a liquid medium that does not, or only to a very small extent, mixes with any of the components of the dispensed fluid; applying a layer of a relative non-volatile inert sealant such as liquid paraffin or silicone or mineral oil, or a combination of these techniques or with other sealing techniques elsewhere described here-in.

25 The final combination of the biomolecule and the components in the cell can be in a liquid, gel or glassy state. In order to induce crystallization in a glassy state, it may be required to raise the temperature or in another way speed up the diffusion of the compound. During crystal growth in a gel, gravity-induced convection is abolished that is caused by local concentration
30 gradients that result from the accretion of molecules on the growing crystal. In

small volumes, this gravity-induced convection is far less severe, since next to the diffusion rate and the speed of crystal growth, it increases with the physical size of the container in which crystallization takes place.

Preferably, the method is carried out in an array of separate cells,
5 whereby each cell contains a different composition. When a change in phase behaviour occurs, this is detected and can be correlated to the specific composition and conditions under which the screening is taking place. However, for instance to provide for an increased reliability of the method, each combination may be provided in duplo or triplo on the same solid support
10 medium.

Any crystal that does not have a cubic crystal structure is known to change the polarization of linearly polarized light, depending on its orientation with respect to the direction of polarization of the through-falling light. This property of crystals and crystalline biomaterial in particular, is at present
15 being used to determine and inspect the quality of individual crystals. However, this property can also be used to determine the presence or absence of a crystal of a biomolecule that is non-cubic. By measuring the change in the polarization of the light falling through, as the direction of polarization is changed, the presence of non-cubic crystalline material can be determined.
20 Preferably the change in phase behaviour of the biomolecule is caused by the formation of crystalline material from the biomolecule.

The crystalline material comprises non-cubic, preferably single crystals, polymorphic crystals, or comprises co-crystals of two or more biomolecules or co-crystals from a biomolecule and a cofactor, complexant or an inhibitor or a
25 salt or a combination thereof.

Detecting changes in phase behaviour can be determined in various ways, but for the rapid screening of multiple samples under reproducible circumstances preferred techniques are based on the detection or measurement, continuously or intermittently, of optical and/or diffraction
30 characteristics of the individual cells.

The determination of the optical and/or diffraction characteristics of the individual cells can be done with a system comprising:

- a polarisable light source
- a holder for
- 5 - a substrate comprising a multitude of cells
- a detecting system for detecting a change in the polarization of the light coming from the polarisable light source.
- a position sensitive detector.

A change in the polarization of the light generally corresponds to a
10 change in phase behaviour. It is preferred that the change in phase behaviour is mainly due to a phase change of the biomolecule. A change in phase behaviour that is not caused by a phase change of the biomolecule may additionally provide useful and valuable information regarding the phase behaviour of the biomolecule itself. For example, if any salt crystallizes out of
15 solution, whilst the substance of interest remains soluble, further the substance of interest cannot be precipitated at its present concentration with said salt.

Detecting phase changes can also be done by visual screening, for instance with the aid of microscopical techniques.

20 A further aspect of the invention pertains to an apparatus for screening the phase behaviour of at least one biomolecule comprising:

- means for holding and supporting a plurality of cells;
- means for providing said cells with a biomolecule;
- means for providing other components to said cells whereby the total
25 composition in the cell has a volume of at most 100 nanoliter;
- means for providing modified conditions to said cells;
- means for detecting changes in phase behaviour of the compositions in said cells.

By combination of the information obtained from the screening,
30 information regarding the phase behaviour is obtained and optimized

conditions for protein crystallogenesi are obtained. In a preferred embodiment, the invention additionally comprises a step wherein detected phase changes are correlated to the combination of compositions and conditions of individual cells. In a further preferred embodiment, these steps
5 are automated, whereby the cells of the substrate are filled with various small volumes of solutions, containing biomolecules, buffers, precipitants, and other components, analogously to a color ink jet printer that applies small volumes of various colors of ink on paper, whereby the identification and screening of crystals does not require manual intervention other than putting the substrate
10 to that analyzer.

Other aspects of the invention relate to the use of nanocrystallisation techniques of the present invention for the preparation and purification of biomolecules or complex organic molecules and for determining the structural characteristics of biomolecules. The method can also be employed for
15 determining the behaviour in solution of the biomolecules.

Description of the Figures:

Figure 1: Nano-wells in elastomer.

Figure 2: Nano-wells in photoresist

20 Figure 3: A lysozyme crystal grown out of 1 nL of mother liquor in a 1nl sized well on an elastomer substrate.

Figure 4: Set-up for automatic identification of non-cubic crystals.

Figure 5: A lysozyme crystal grown out of 3 nL of mother liquor on a glass substrate, after dispensing the protein solution using a piezo-electric
25 dispensing robot

Figure 6. A lysozyme crystal obtained from a screen dispensed using a piezo-electric dispensing robot.

Examples

I. Preparation of substrates contain small wells

5 Substrates containing 1 nL wells were manufactured in two fashions from two different materials:

Ia. Elastomer substrate

10 A circular brass mold (diameter 20 mm) was micro-machined with a CNC-cutting machine to have four square arrays of 21 by 21 cubic protrusions of 100 by 100 by 100 mm each. Subsequently the elastomer, Sylgard 184 (manufactured by Dow Corning), was mixed with the supplied catalyst according the manufacturers protocol, mixed by swirling and poured into the mold and degassed under vacuum. Polymerization of the elastomer was
15 accelerated by storing the mold with liquid elastomer in an oven at 80 °C. The polymerized elastomer was removed from the mold after cooling to ambient temperature. Figure 1 shows several 100 mm wells in elastomer.

Ib. Photoresist substrate

20 A layer of 50 - 100 µm of NANO™ XP SU-8 50 negative photoresist (manufactured by MicroChemCorp, Newton, MA) was spin coated on a (76.0 mm x 25.4 mm x 1 mm) carefully pre-cleaned glass microscope objective slide. It was pre-baked for 30 minutes at 95 °C in an oven. After a relaxation period of 3 hours the dried photoresist was covered with a computer generated
25 pattern, transferred to a photographic negative. After a 30 to 120 seconds near-UV illumination a post-bake of 30 min at 95 °C was applied before the non-exposed parts of the pattern were washed away with XP SU-8 developer for 5 minutes. The material was subjected to a final hard-bake at 150 °C
30 overnight. Figure 2 shows several 100 mm wells in photoresist, part of an array of 100 x 100 wells in a rectangular configuration.

II. Method of filling of nanoliter sized wells

The filling arrays of nanoliter sized wells in a hydrophobic substrate with hydrophilic solutions requires non-standard techniques. Two methods for filling elastomer substrates and photoresist substrates are described.

II.a Flood filling

The elastomer substrate with four arrays of 21 by 21 nL sized wells was flushed with 500 ml of pure methanol, wetting the nL sized wells, followed by flushing with methanol diluted with water, and then by water. After that most of the water was evaporated.

A protein solution that had been filtered with a low protein binding membrane with a nominal cut-off of 0.2 μ m and contained 40 mg/ml of lysozyme from hen egg white (manufacturer Boehringer Mannheim), 0.02 M sodium acetate pH 4.7 and 5% sodium chloride was layered on the wetted wells.

To prevent rapid evaporation in the nL wells, paraffin oil was layered on top of the lysozyme solution. Excess of the lysozyme solution outside the nL sized wells was carefully removed by a combination of blotting with standard filter paper (manufacturer Whatman) and wiping with a glass capillary with an external diameter of 50 μ m. The paraffin oil covered substrate was covered with a standard microscope glass cover slide (22 mm x 22 mm).

IIb. Nano-dispensing

Individual droplets with a volume of approximately 70 picoliter consisting of a solution of a red dye (Ponceau S, P-3504, (3-hydroxy-4-[2 sulfo-4-(4-sulfo-phenylazo)phenylazo]-2,7-naphthalenedisulfonic acid) from Sigma Chemicals) in water were deposited into the individual nL sized wells of an array of 100 by 100 wells (as described previously) in a photoresist substrate using a MicroDrop nano-dispensing station (manufacturer MicroDrop GmbH).

The red dye allowed inspection with a microscope of the individual droplets and confirmed that they had indeed only been deposited in the wells and not elsewhere. Subsequently other liquids were deposited in a similar fashion.

5 III. Protein crystallization in nanoliter volumes.

The same mother liquor, containing 40 mg/ml of lysozyme from hen egg white (manufacturer Boehringer Mannheim), 0.02 M sodium acetate pH 4.7 and 5% sodium chloride that in a comparative experiment had resulted in protein crystals in a bulk assay was now used to perform crystallization in the
10 elastomer substrate. After filtration and application to the elastomer substrate using the flood-filling technique, protein crystals were grown in a number of nL wells in a period of one to two days during storage in a temperature controlled environment at 25 °C. The results are displayed in Figure 3.

15 IV. Automatic identification of non-cubic crystals in large arrays.

A set-up was constructed as depicted in Figure 4. An elastomer substrate containing 1 nL wells, some of which contained lysozyme crystals was placed between two polarizing filters at 90° relative to each other (the 'polariser' and the 'analyzer' filters). The light source, polarizing filters,
20 stereomicroscope and CCD detector were manufactured by Leica. Either the sample, or simultaneously the two polarisers were rotated, and wells containing crystals were identified optically as those positions on the substrate where light was transmitted and subsequently extinguished, depending on the angle of the crystal axes relative to the direction of polarization of the polariser
25 filter.

The combination of integrating the transmitted light using an optical area detector, together with the coupled rotation of the pair of polarisers forms the essential feature of this method. Non-cubic crystalline material can be identified in positions that show a high variation in transmitted light, relative
30 to the orientation of the crystal axes. The minimum size of typical protein

crystals that can be identified in this manner is substantially smaller than 5 μ m. A field of view of at least 10 by 10 nL sized wells can be inspected simultaneously.

5 V. Comprehensive mapping of the phase behaviour of biomolecules in 1000 or more simultaneous tests for identifying crystallization conditions.

Here an example is given of the importance of comprehensive mapping of phase space. This mapping can also be attained by using other buffering components and/or other precipitants. Preferably as many different conditions
10 as possible are to be tested. Usually in odds with this observation, using a substance that is as pure as can be obtained also contributes to success.

To identify crystallization conditions for porcine β -lactoglobulin, the following buffers were prepared:

B1 (80.3 mmol of formic acid + 19.6 mmol of Na-formate, add H₂O to 100
15 ml, adjust pH to 3.0);

B2 (55.1 mmol of formic acid + 44.8 mmol of Na-formate, add H₂O to 100 ml, adjust pH to 3.5);

B3 (52.2 mmol Citric acid + 47.8 mmol Na-citrate, add H₂O to 100 ml, adjust pH to 4.0);

20 B4 (55.7 mmol Acetic acid + 44.2 mmol Na-acetate, add H₂O to 100 ml, adjust pH to 4.5);

B5 (28.3 mmol Hac + 71.6 mmol NaAc, add H₂O to 100 ml, adjust pH to 5.0);

B6 (19.52 gr MES, add H₂O to 100 ml, adjust pH to 5.5);

25 B7 (19.52 gr MES, add H₂O to 100 ml, adjust pH to 6.0);

B8 (19.52 gr MES, add H₂O to 100 ml, adjust pH to 6.5);

B9 (23.83 gr. HEPES, add H₂O to 100 ml, adjust pH to 7.0);

B10 (23.83 gr. HEPES, add H₂O to 100 ml, adjust pH to 7.5);

B11 (12.14 gr. of TRIS, add H₂O to 100 ml, adjust pH to 8.0);

B12 (6.32 gr. of BICINE, add H₂O to 100 ml, adjust pH to 8.5);

B13 (6.32 gr. of BICINE, add H₂O to 100 ml, adjust pH to 9.0);

B14 (22.13 gr. of CAPS, add H₂O to 100 ml, adjust pH to 10.0).

MES = 2-[N-morpholino]ethanesulphonic acid; HEPES = N-[2-

5 hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]; Tris =
tris(hydroxymethyl)aminoethane; Bicine = N,N-bis[2-hydroxyethyl]glycine;
CAPS = 3-cyclohexylamino]-1-propanesulphonic acid

Subsequently the following precipitant stock solutions in H₂O were
10 prepared:

P1 (4.0 M ammonium sulphate);

P2 (5.0 M sodium chloride);

P3 (10 M lithium chloride);

P4 (1.6 M Na,K tartrate);

15 P5 (100% PEG 400);

P6 (50% PEG 2000);

P7 (50% PEG 4000);

P8 (50% PEG 8000);

P9 (30% PEG 20,000);

20 P10 (50% Jeffamine M-600);

P11 (100% ethylene glycol);

P12 (50% dioxane).

Then, using an IMPAX pipetting robot from Douglas Instruments, the
25 following mixtures were prepared in 11 standard 96-wells plates: 0.5 microliter
β-lactoglobulin (25 mg/ml in 0.005 mM Tris-HCl pH 7.8), to which 0.1
microliter of buffer stock was added (either B1, B2, B3, B4, B5, B6, B7, B8, B9,
B10, B11, B12, B13, or B14). To this was added either 0.15, 0.20, 0.25, 0.30,
0.35, or 0.40 microliter of precipitant solution, diluted with either 0.25, 0.20,
30 0.15, 0.10, 0.05, or 0.00 microliter of H₂O, respectively. In total 1008 different

conditions were therefore tested simultaneously (all permutations of 14 different buffers and 12 different precipitants, the latter at 6 different concentrations). Evaporation of the crystallization tests was slowed down by layering 1 mm of a 9:1 mixture of paraffin oil and silicon oil over the
5 crystallization set-ups.

From the screen it was evident that porcine β -lactoglobulin does not crystallize above pH 4.0 with any precipitant in any condition. Between pH 3.0 and 4.0, this protein only crystallizes with NaCl as precipitant in concentrations of 0.75-1.25 M, between 10 °C and 20 °C. Optimal
10 crystallization conditions were subsequently found to be: 100mM formate buffer pH 3.2; 1.3 M NaCl; 12.5 mg/ml β -lactoglobulin; 20 °C. These crystals diffract to 2.3 Å on a rotating anode X-ray source. The structure was solved from SIRAS phases in less than 2 months after setting up the initial crystallization trials. Crystallization conditions were not found using the
15 widely used commercially available sparse matrix kits from Hampton Research, 27632 El Lazo Road, Suite 100, Laguna Niguel, CA 92677-3913 USA. In contrast, bovine β -lactoglobulin, sharing 66% amino acid identity with porcine β -lactoglobulin, crystallizes in at least 6 different crystal forms, with a pH ranging from 6.0 to 8.5 (Qin et al, Biochemistry 37:14014-23, 1998).

20

VI. (Limited) screening of the phase behaviour of lysozyme in an array after dispensing with a piezo-electric dispensing robot

Here an example is given that demonstrates the feasibility of dispensing an array of cells, containing the protein lysozyme in a multitude of
25 compositions with different buffers and/or different precipitant concentrations. For this experiment we used a lysozyme solution of: 20 mg/mL in filtered demineralized water. The precipitant stock solution used was a 3M NaCl solution in filtered demineralized water. We used the following buffer stocks:

	buffer B1 :	1M NaAc	pH 4.0
	buffer B2 :	1M NaAc	pH 4.6
	buffer B3 :	1M Na-citrate	pH 5.6
	buffer B4 :	1M HEPES	pH 7.0
5	buffer B5 :	1M TRIS	pH 7.4

Using a piezo-electric dispensing system that dispenses 100 picoliter droplets, we pipetted the following scheme, directly through a 1 mm sealant layer of paraffin oil, in a 15 x 15 well array:

10 All wells of rows A-C received a constant volume of 16 nl (160 drops) buffer B1:

All wells of rows D-F received a constant volume of 16 nl buffer B2:

All wells of rows G-I received a constant volume of 16 nl buffer B3:

All wells of rows J-L received a constant volume of 16 nl buffer B4:

15 All wells of rows M-O received a constant volume of 16 nl buffer B5:

Each row received a gradient volume of precipitant stock solution: Start 0 nL, increment 2.4 nL (24 drops) per well

Each row received a gradient volume of filtered demineralized water: Start 33.6 nL, increment -2.4 nL per well. The final NaCl concentration varied
20 between 0 and 1 M in each row.

Each well finally received 48 nL (480 drops) of protein solution, final volume in all wells was less than 100 nL.

Lysozyme, as expected, crystallises best in wells containing buffer B2, at a NaCl concentration around 0.4M. Crystals grew in 24 hours. The protein
25 forms an amorphous precipitate at lower and higher pH, as well as at high NaCl concentrations.

The experiments, dispensed in triplo, showed very good reproducibility.

Claims

1. A method for identifying crystallization conditions for at least one biomolecule, said method comprising:
 - providing a set of compositions of a volume of 1-100 nanoliter, composition of said set comprising said at least one biomolecule;
 - 5 inducing or allowing each said composition to adopt at least a first condition possibly influencing said crystallization; and
 - detecting crystallization in said composition.
2. The method according to claim 1, wherein said composition differs in said first condition from any other compositions of said set.
- 10 3. The method according to claim 1, wherein said first condition comprises a change in a physical condition.
4. The method according to claim 1, wherein said first condition comprises a chemical condition.
5. The method according to claim 1, wherein said set of compositions is
- 15 provided on a solid support medium.
6. The method according to claim 5, wherein said solid support medium comprises a multitude of separate cells, each comprising one composition.
7. The method according to claim 1, further comprising changing at least a first condition after exposing said composition with said first condition.
- 20 8. The method according to claim 1, further comprising identifying conditions under which desired size and homogeneity of crystals of said at least one biomolecule is obtained.
9. The method according to claim 1, wherein said at least one biomolecule is a proteinaceous molecule.
- 25 10. The method according to claim 1, wherein said biomolecule is selected from the group consisting of proteins, peptides, protein containing complexes, enzymes, enzyme complexes, nucleic acids, or nucleic acid containing complexes or chemically modified forms and mixtures thereof.

11. The method according to claim 1, wherein the biomolecule is provided in the form of a crystalline or aggregated state, solution, emulsion or a suspension.
12. A method for producing a composition comprising crystals of a biomolecule of a narrow size distribution, said method comprising:
- 5 selecting crystallization conditions for said biomolecule providing a set of compositions of a volume of 1-100 nanoliter, composition of said set comprising said at least one biomolecule;
- inducing or allowing each said composition to adopt at least a first
- 10 condition possibly influencing said crystallization;
- detecting crystallization in said composition; and
- scaling up said conditions to a desired total volume.
13. The method according to claim 12, wherein said composition comprises crystals of a biomolecule of a narrow size distribution obtained by a method
- 15 according to claim 12.
14. The method of claim 12, wherein said composition is used as a source for crystallization nuclei in a crystallization process for said biomolecule.
15. The method of claim 12, wherein said method comprises providing an over-saturated composition of said biomolecule.
- 20 16. The method of claim 12, wherein the structure of said biomolecule is determined by X-ray diffraction.
17. The method of claim 12, wherein the structure of said biomolecule is determined by electron diffraction.
18. A method for screening the phase behaviour of at least one biomolecule
- 25 comprising:
- providing a solid support medium comprising a multitude of separate test positions;
- providing at least two of the separate test positions with a solution of said biomolecule and other components such that the total composition in said
- 30 separate test position differs in at least one aspect from the total composition

of other test positions and the total composition in the test position has a volume of at most 100 nanoliter; and

detecting changes in phase behaviour of the compositions in the separate test positions.

5 19. The method according to claim 18, wherein the conditions that lead to changes in phase behaviour in individual compositions are modified.

20. The method according to claim 18, wherein the composition of the test position is further modified.

10 21. The method according to claim 18, wherein a detected phase change in said composition is compared with the other compositions.

22. The method according to claim 18, wherein the change in phase behaviour of the composition is predominantly caused by a phase change of the biomolecule.

15 23. The method according to claim 18, wherein the surface of the solid support medium comprises at least one crystallization nucleus comprising an inert crystal.

24. The method according to claim 18, wherein the composition comprises at least one crystallization nucleus comprising an inert crystal.

20 25. The method according to claim 18, wherein the test positions have a volume or are filled to a total volume of at most 50 nanoliter.

26. The method according to claim 18, wherein said test positions have a volume or are filled to a total volume of at most 1 nanoliter.

27. The method according to claim 18, wherein said test positions have a volume or are filled to a total volume of at most 100 picoliter.

25 28. The method according to claim 18, wherein said test positions have a volume or are filled to a total volume of at most 10 picoliter.

29. The method according to claim 18, wherein said test positions contain at most 100 ng of biomolecule.

30 30. The method according to claim 18, wherein said test positions contain at most 10 ng of biomolecule.

31. The method according to claim 18, wherein said changes in phase behaviour are detected by measuring optical characteristics of the separate test positions.
32. The method according to claim 18, wherein said biomolecule and
5 optional other components are added to the test positions by means selected from the group consisting of piezo-electric dispensing techniques, bubble jet dispensing techniques, electrospray dispensing techniques, micro- and nano-dispensing techniques, or flood filling or combinations thereof.
33. The method according to claim 32, wherein said biomolecule and
10 optional other components are added to the test positions through a layer of liquid that is immiscible with the compositions in said test positions.
34. The method according to claim 18, wherein the biomolecule is selected from the group consisting of proteins, peptides, protein containing complexes, enzymes, enzyme complexes, nucleic acids, or nucleic acid containing
15 complexes or chemically modified forms and mixtures thereof.
35. The method according to claim 18, wherein said biomolecule is provided in the form of a crystalline or aggregated state, solution, emulsion, or a suspension.
36. The method according to claim 18, wherein the change in phase
20 behaviour of the biomolecule comprises the formation of crystalline material of the biomolecule.
37. The method according to claim 36, wherein the crystalline material of the biomolecule comprises polymorphic crystals.
38. The method according to claim 36, wherein the crystalline material of
25 the biomolecule comprises co-crystals of two or more biomolecules.
39. The method according to claim 36, wherein the crystalline material of the biomolecule comprises co-crystals of a biomolecule with a small molecule.
40. The method according to claim 39, wherein said small molecule is a pharmaceutical.

41. Localization of non-cubic crystalline material on the substrate by monitoring local changes in polarization, that are dependent on the direction of polarization of through-falling light, whereby said direction is changed.

42. A method for screening the phase behaviour of at least one biomolecule
5 comprising:

providing a solid support comprised of a multitude of separate test positions;

providing at least two of said separate test positions with a solution of said biomolecule and other components such that the total composition in said
10 test positions differs in at least one aspect from said total composition of other test positions and said total composition in said test position has a volume of at most 100 nanoliter;

modifying the conditions that lead to changes in phase behaviour in individual compositions;

15 inducing nucleation; and

detecting changes in phase behaviour of said compositions in said separate test positions.

43. The method according to claim 42, wherein nucleation is induced by agitation, vibration, micro-wave treatment, (ultra)sonic treatments or a
20 combination thereof.

44. The method according to claim 42, wherein nucleation is induced by the adding of nucleation centres.

45. The method according to claim 44, wherein said nucleation centers are in the form of small crystallites.

25 46. The method according to claim 45, wherein the small crystallites are salts or complexes of the biomolecule.

47. A method for the preparation of a solid support medium containing wells with a volume up to 100 nanoliter for nanocrystallisation comprising:

providing a mold with protrusions having the desired size and shape;
30 applying an elastomeric composition to the mold; and

polymerizing the elastomeric composition.

48. A method for the preparation of a solid support medium containing wells with a volume of up to 100 nanoliter comprising:

providing a layer of negative photoresist on a carrier body;

5 applying a pattern to said photoresist;

applying UV-illumination to the photoresist; and

removing the non-illuminated parts of the photoresist.

49. An apparatus for screening the phase behaviour of at least one species of biomolecule comprising:

10 means for holding and supporting a plurality of test positions;

means for providing said test positions with a biomolecule;

means for providing other components to said test positions whereby the total composition in the test position has a volume of at most 100 nanoliter;

means for providing modified conditions to said test positions; and

15 means for detecting changes in phase behaviour of the compositions in said test positions.

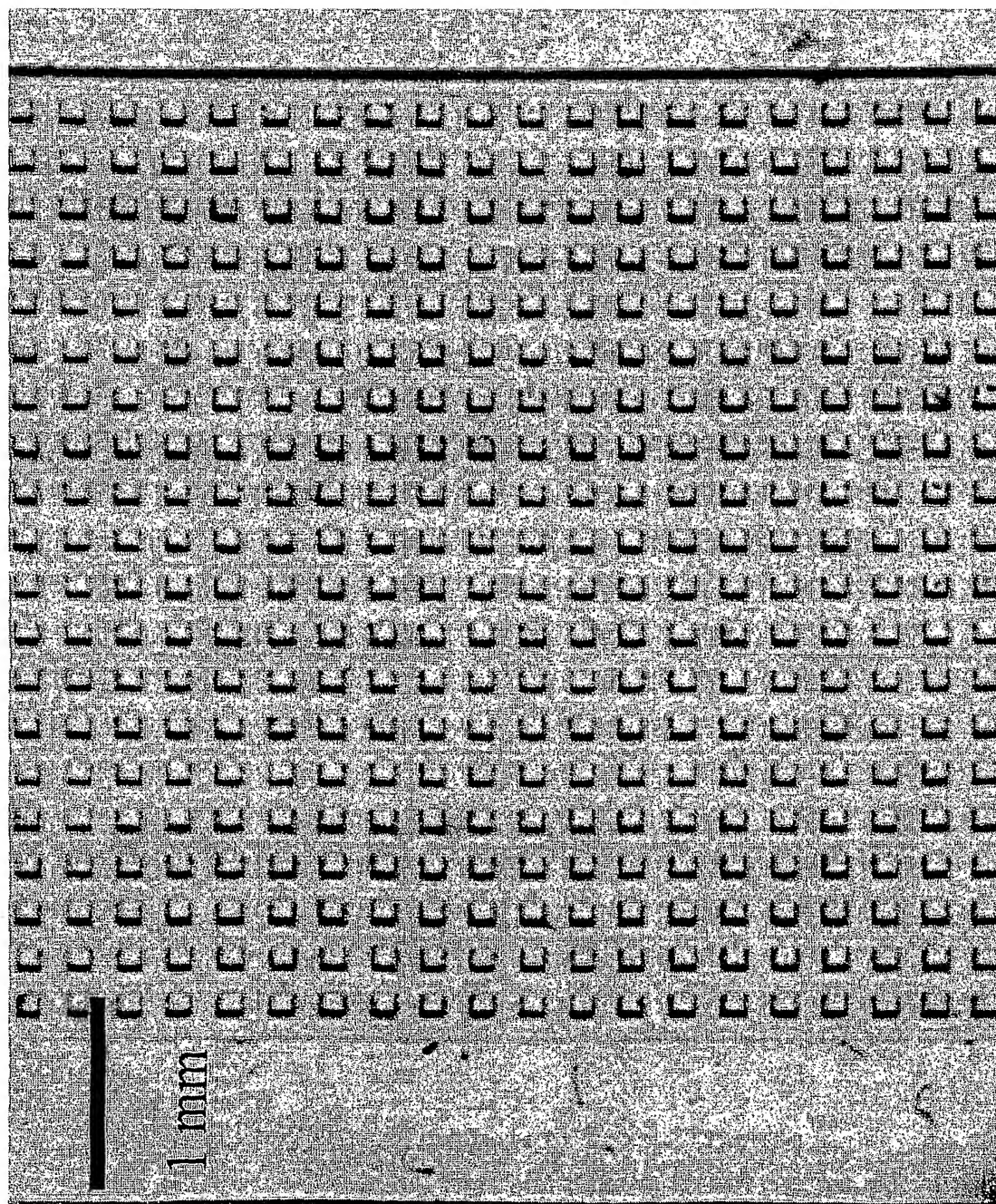


Figure 1: 400 nano-wells in elastomer

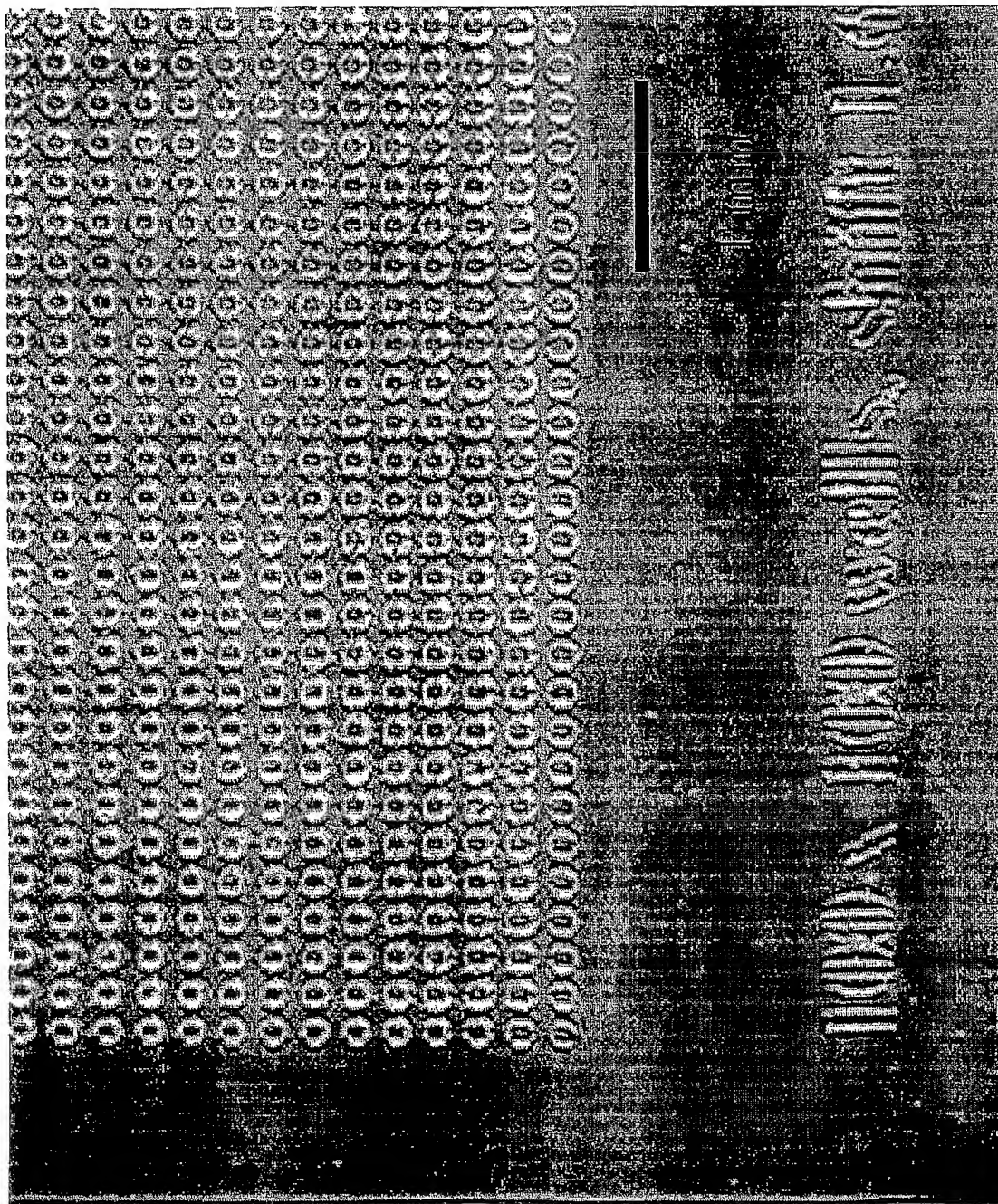


Figure 2: 378 nano-wells in photoresist

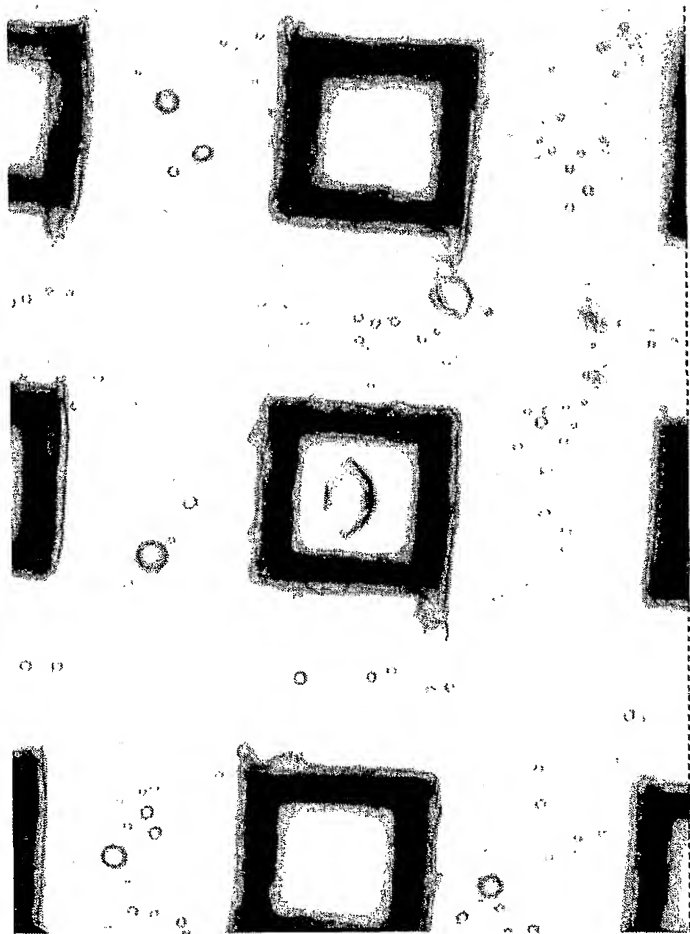


Figure 3: A lysozyme crystal grown out of 1 nl of mother liquor in a 1 nl sized well on an elastomer substrate

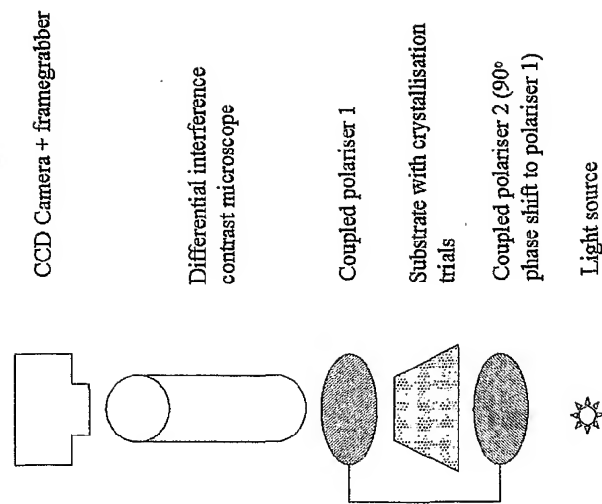


Figure 4: Set-up for automatic identification of non-cubic crystals



Fig. 5

6/6

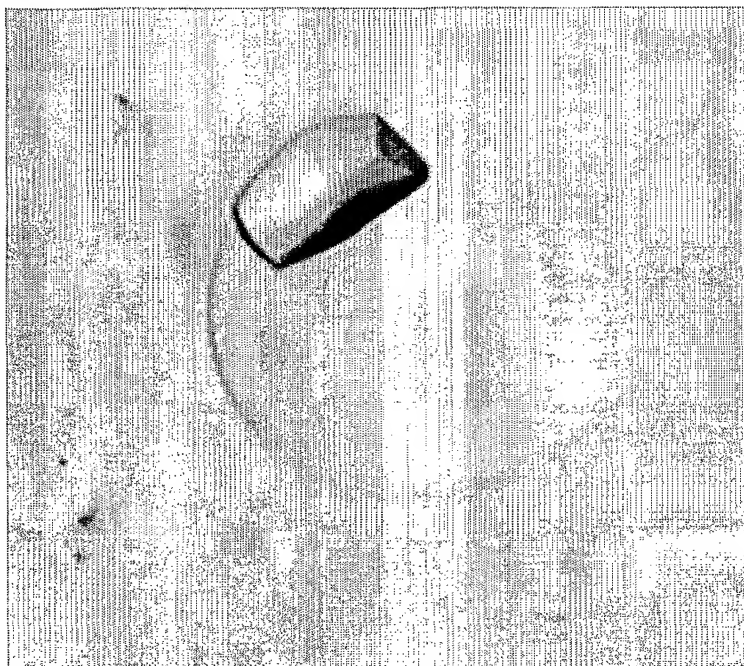


Fig. 6